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Link between Culture Zeta Potential Homogeneity and Ebp in *Enterococcus faecalis*

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***Enterococcus faecalis*, a commensal of the gastrointestinal tract and an opportunistic pathogen, has the ability to adhere to surfaces and form biofilms. It has been shown earlier that only 10 to 20% of an *E. faecalis* OG1RF culture expresses endocarditis- and biofilm-associated pili (Ebp), which are involved in biofilm formation. Another study revealed that *E. faecalis* clinical isolates, as well as OG1RF, are heterogeneous with respect to their apparent zeta potential, which was also correlated with increased ability to form biofilm. The aim of this study was to demonstrate that the heterogeneity in the presence of Ebp is correlated to that in apparent zeta potential. Heterogeneous cultures of OG1RF showed two distinct subpopulations with the most (−38 mV) and least (−26 mV) negative zeta potential. Deletion of EbpR, the activator of the *ebp* operon, or the structural genes *ebpABC* resulted in homogeneous culture with the most negative zeta potential. Conversely, overexpression of EbpR or the structural genes *ebpABC* resulted in homogeneous culture with the least negative zeta potential. The results show that *ebp* operon expression in *E. faecalis*, as measured by using *P_{ebp}-gfp* promoter fusion, is the cause of heterogeneity in zeta potential and that pilus production causes the cells to behave as the least negative particle in an electric field.**

Enterococcus faecalis is a Gram-positive commensal of the gastrointestinal tract in both humans and animals that has the ability to become pathogenic when there is an opportunity. *E. faecalis* is responsible for 80 to 90% of human enterococcal infections (11, 12). *E. faecalis* has become increasingly hard to treat due to increasing occurrence of antibiotic resistance (24). In the late 1980s, three vancomycin-resistant strains of *E. faecalis* were described (25), of which the genome of one, strain V583, has been sequenced recently (24). In addition, *E. faecalis* is able to form biofilms that are covered by an extracellular polysaccharide matrix (23). This self-produced extracellular matrix makes microbial biofilms notoriously hard to eradicate, since they can withstand antibiotics at 10 to 1,000 times the concentration required to kill planktonic bacteria (16). Enterococci have been reported to form biofilms on several types of medical devices such as intravascular catheters (26), biliary stents (7), urethral stents (13), and silicone and gastrostomy devices (6). They are also able to form biofilms on living tissue such as heart valves, causing native valve endocarditis (20).

Because of its pathogenicity and biofilm forming ability, *E. faecalis* has become a point of focus and recent genetic studies have identified several genes that are involved in biofilm formation by *E. faecalis* (2, 15, 17), such as those in the endocarditis- and biofilm-associated pilus operon (*ebpABC*) (15, 20). This operon, present in the majority of enterococcal isolates from clinical and nonclinical origin (1), is followed by a sortase gene (*srtC*) that has its own promoter. SrtC binds EbpA, EbpB, and EbpC together to form a pilus-like structure on the outer cell surface (14, 20). The *ebpABC* operon is positively regulated by *ebpR*, which is located upstream of and in opposite orientation to the *ebpABC* operon (3). The intergenic region contains both the promoters for *ebpR* and *ebpABC*. Disruption of *ebpA*, *ebpB*, or *ebpC* reduced biofilm formation to a great extent (20). In the same study, it was shown that only 10 to 20% of the cells in a culture displayed Ebp pili on

their surface. Ebp-type pili are highly conserved in *E. faecalis* and mediate adherence to platelets, fibrinogen, and collagen (19, 21).

Although a bacterial culture is often considered a population of identical organisms, it is becoming increasingly clear that subpopulations with different properties exist, e.g., competent and noncompetent cells or sporulating and nonsporulating cells in cultures of *Bacillus subtilis* (8, 30). The presence of such culture heterogeneity has also been observed in many *E. faecalis* strains with respect to the apparent zeta potential (28, 29). In fact, there are two types of clinical isolates, namely, those that are homogeneous and those that form heterogeneous cultures with respect to apparent zeta potential. Strains that are heterogeneous in apparent zeta potential adhere better to surfaces than homogeneous strains, although the initial adhesion rates were the same for both types of strains. Heterogeneity in apparent zeta potential was also correlated with the ability to form biofilms, as homogeneous strains showed a significant reduction in biofilm formation. The distribution of the subpopulations in cultures of heterogeneous strains is strain dependent; in two of the investigated *E. faecalis* strains, the smaller (25%) subpopulation contained cells with the most negative apparent zeta potential (strains BS385 and BS1037), while in two other strains, the smaller subpopulation represented cells with the least negative apparent zeta potential (strains BS4126 and BS12297) (28, 29).

The zeta potential is commonly used to calculate the surface charge of colloids or bacteria (31). In suspension, there are two liquid layers around every particle of which the inner or Stern

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TABLE 1 Strains, plasmids, and primers used in this study

Strain or plasmid	Specification or description ^a	Reference	Oligonucleotide	Sequence (5' to 3') ^b
Strains				
OG1RF	Wild type	9	EbpA pro pMV158 fw	CGCGGGT <u>ACC</u> ATTGTTTCTCCCAAGTCCTTTAC
1D21 (G9)	OG1RF <i>ebpR::EfaMarTn (ebpR::Tn)^c</i>	15	EbpA pro pMV158 rv	CGCTCTAGAGTTGAGCGGATGTAATTTCAACTTC
OG1RFΔ <i>ebpABC</i>	OG1RF clean knockout of <i>ebpABC</i>	21	pEbpA pNZ fw	GCGCAGATCTCGATAATTTTTTCTAAAAACCTAATCATATTG
TX5662	OG1RF Δ <i>ebpABC</i> (pAT392:: <i>ebpABC</i>)	21	pEbpA pNZ rv	GCGCGAATTTCATCATCTCATCTGTTATCATTTG
Plasmids				
pMSP3535	Em ^r ; nisin-inducible gene expression vector, carrying pAMB1 and ColE replicons, <i>nisRK</i> , P _{<i>nisA</i>}	5		
pMSP3545	Em ^r ; nisin-inducible gene expression vector, carrying pAMB1 and ColE replicons, <i>nisRK</i> , P _{<i>nisA</i>}	5		
pCJK55	Mariner transposase C9 cloned downstream of P _{<i>nisA</i>} in pMSP3545	15		
pCJK128	<i>ebpR</i> cloned downstream of P _{<i>nisA</i>} in pMSP3535	15		
pMV158GFP	Tet ^r ; carrying <i>PmalT::gfp</i>	22		
pMT02	Tet ^r ; pMV158 carrying P _{<i>ebp</i>} :: <i>gfp</i>	This study		

^a Em^r, erythromycin resistance; Tet^r, tetracycline resistance.^b Sites for restriction enzymes are underlined.^c Abbreviated nomenclature.

layer lies just around the surface and contains ions that are strongly bound to the surface. Since the surfaces of bacteria are almost always negatively charged, the ions in the Stern layer are positively charged. Outside of the Stern layer is an electrical double layer in which both positive and negative ions can be found. These ions are not bound tightly, but when the particle (cell) moves, this layer also moves. The ions beyond the electrical double layer stay dispersed in the liquid. The potential on the boundary of the electrical double layer and the surrounding liquid bulk is the zeta potential, which can be measured using microelectrophoresis (18, 31). The apparent zeta potential is dictated by the charges on the cell surface as well as the presence of surface structures that influence the width of the double layer (the softness of the particle) (27) and is calculated from the electrophoretic mobility of the particle in an electric field. Thus, heterogeneity in pilus expression (20) could be the cause of the observed heterogeneity in the apparent zeta potential of enterococcal isolates (29), a supposition that was further examined in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. All *E. faecalis* strains were grown at 37°C in M17 (Difco) broth with 0.2% glucose (GM17) and, when appropriate, 100 μg/ml erythromycin or 10 μg/ml tetracycline for *Escherichia coli* and 10 μg/ml erythromycin or 4 μg/ml tetracycline for *E. faecalis* strains. *E. coli* was grown at 37°C in tryptone-yeast extract (TY) medium (10 g liter⁻¹ tryptone, 5 g liter⁻¹ yeast extract, 5 g liter⁻¹ NaCl [pH 7.2]). Expression was induced with nisin (25 ng/ml; nisin not milk solid) at an optical density (OD) at 600 nm of 0.3. Nisin (N-5764), which is supplied in milk solid containing 2.5% nisin by weight, was obtained from Sigma. Stock solutions were made by dilution in deionized water.

DNA preparations and transformation. Plasmid DNA from *E. coli* was isolated by the High Pure plasmid isolation kit (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Other methods, including DNA restriction and ligation, purification, and agarose gel electrophore-

sis, were performed according to the guidelines of the enzyme manufacturer or using standard protocols. All restriction enzymes and T4 DNA ligase were obtained from Fermentas (Fermentas GmbH, St. Leon-Rot, Germany) or from Roche Diagnostics Nederland B.V. The sequences of the oligonucleotides used in this work are listed in Table 1.

Electrocompetent cells were obtained by growing *E. faecalis* strains in GM17 medium with 1% glycine and 0.5 M sucrose using the protocol as described for *Lactococcus lactis* by Holo and Nes (10). *E. coli* cells were grown in TY medium to an OD at 600 nm of 0.3 to 0.5 and washed three times with ice-cold water. Electrotransformation of competent cells was performed using the Gene Pulser (Bio-Rad, Richmond, CA) with standard settings, using 2.25 kV for *E. coli* and 1.6 kV for *E. faecalis*. After electrotransformation, *E. faecalis* was incubated for 2 h at 37°C in recovery medium (GM17, 0.5 M sucrose, 20 mM MgCl₂, 2 mM CaCl₂, and, when required, 50 ng/ml erythromycin) and *E. coli* in TY for 1 h at 37°C with shaking at 200 rpm.

Construction of an *ebp* promoter-*gfp* fusion. The *ebpABC* operon promoter P_{*ebp*} was fused to the green fluorescent protein gene *gfp* from *Aequorea victoria*, using vector pMV158GFP (22). The maltose promoter driving GFP expression in this vector was replaced by P_{*ebp*} as follows: the whole intergenic region of 327 bp between *ebpA* and *ebpR* of *E. faecalis* OG1RF, containing the two oppositely oriented promoters P_{*ebp*} and P_{*ebpR*}, was amplified by PCR using primers EbpA pro pMV158 fw and EbpA pro pMV158 rv (Table 1). The PCR product and the vector were restricted with KpnI and XbaI and subsequently ligated using T4 DNA ligase. The proper construct was obtained in *E. coli* DH5α and was confirmed by PCR and DNA sequencing. In this vector, named pMT02, P_{*ebp*} is fused to *gfp* (Fig. 1). We introduced pMT02 in *E. faecalis* OG1RF strains with various mutations in the *ebp* locus (15, 21).

Flow cytometry analyses. P_{*ebp*}-driven expression of GFP was examined by growing *E. faecalis* strains carrying pMT02 in GM17 for 24 h. Cells were centrifuged to remove the medium and diluted 100-fold in minimal medium and directly measured in a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands) with an argon laser (488 nm). At least 20,000 cells were analyzed for each sample. Data containing the signals were collected using a fluorescein isothiocyanate (FITC) filter and the photomultiplier voltage set between 700 and 800 V.

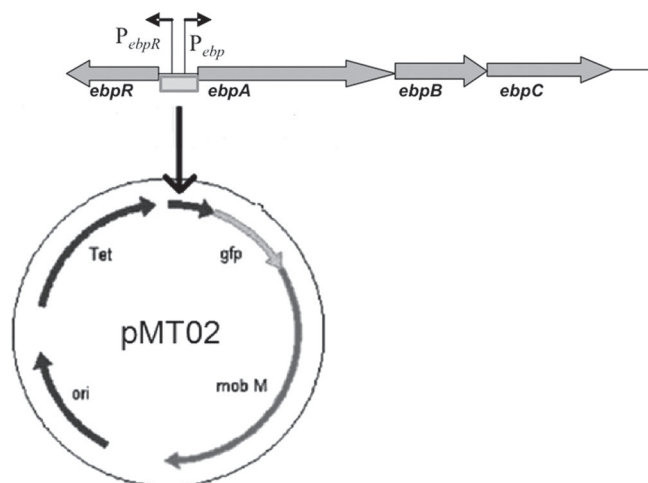


FIG 1 Construction of pMT02. The intergenic region of 327 bp between *ebpA* and *ebpR*, containing the two oppositely oriented promoters P_{ebp} and P_{ebpR} , was amplified by PCR. The fragment was used to replace P_{malT} in pMV158GFP in such a way that P_{ebp} is fused to *gfp*.

Data were captured using EXPO32 software (Beckman Coulter) and further analyzed using WinMDI 2.9 software (<http://facs.scripps.edu/software.html>). Figures were prepared using WinMDI 2.9 and CorelDRAW X3. To distinguish background fluorescence from GFP-specific fluorescence, the parental strain *E. faecalis* OG1RF was used as a control.

Zeta potential measurements. *E. faecalis* strains were grown overnight at 37°C in 10 ml GM17 containing the appropriate antibiotic and induced with nisin at an OD at 600 nm of 0.3, when appropriate. Cells were harvested by centrifugation at $6,500 \times g$ for 5 min at 10°C and washed twice with 10 ml of 10 mM potassium phosphate buffer (pH 7), after which the pellet was resuspended in 1 ml of the same buffer. The cell suspension was sonicated on ice (3×10 s with intervals of 1 min). Apparent zeta potentials were measured with a Lazer Zee model 501 meter (PenKem, Bedford Hills, NY), with camera, by adding a number of drops of the bacterial suspension to 30 ml of 10 mM potassium phosphate buffer (pH 7.0). The diluted suspension was loaded into the cell, after which a voltage difference of 150 V was applied and the velocity of the cells was

observed by the camera. Apparent zeta potentials were calculated from these measurements (28, 29).

Effect of bile on *ebp* expression in *E. faecalis*. All *E. faecalis* strains with or without pMT02 were incubated in GM17 with appropriate antibiotics, in the presence or absence of ox bile (40 mg ml^{-1} ; Merck, Darmstadt, Germany) at 37°C, and with shaking at 100 rpm for 24 h. P_{ebp} -driven GFP expression was measured by flow cytometry as described above.

Cloning and sequencing of *ebpR-ebp* promoter region. Colony PCR was done on several human clinical isolates of *E. faecalis* using the primers pEbpA pNZ fw and pEbpA pNZ rv (Table 1). The pCR 2.1 vector (Invitrogen Life Technologies, Carlsbad, CA) was used as a cloning vector, and PCR fragments were directly ligated in the vector using T4 DNA ligase according to the manufacturer's protocol. After transformation of the ligated product into *E. coli* DH5 α , clones containing the various *ebpR-ebp* promoter regions were picked, verified by restriction enzyme digestion, and commercially sequenced using M13 universal primers (ServiceXS, Leiden, The Netherlands). Resulting DNA sequences were aligned with published sequences and examined using Geneious Pro trial 3.8.5 (<http://emboss.sourceforge.net>).

RESULTS

P_{ebp} promoter activity in heterogeneous *E. faecalis* OG1RF. Expression of the *ebp* operon is positively regulated by EbpR, the gene of which is located in opposite orientation upstream of the operon (3) (Fig. 1). To study the activity of the *ebp* promoter in *E. faecalis* OG1RF, the *ebpR-ebpA* intergenic region from *E. faecalis* OG1RF was amplified and used to replace the maltose promoter upstream of the *gfp* gene in pMV158GFP (22). This was done in such a way that in the resulting plasmid, pMT02, the P_{ebp} promoter drives *gfp* expression. Plasmid pMT02 was introduced in *E. faecalis* OG1RF. GFP expression was examined using flow cytometry in which fluorescence intensity of at least 20,000 *E. faecalis* OG1RF(pMT02) cells was analyzed. In a culture of OG1RF(pMT02), approximately 20% of the cells produced GFP while the control strain without the plasmid did not show detectable fluorescence (Fig. 2). *E. faecalis* OG1RF is heterogeneous with respect to apparent zeta potential at pH 7.0 (28, 29). Plasmid pMT02 did not have an effect on this property, as *E. faecalis* OG1RF(pMT02) indeed showed two populations with respect to

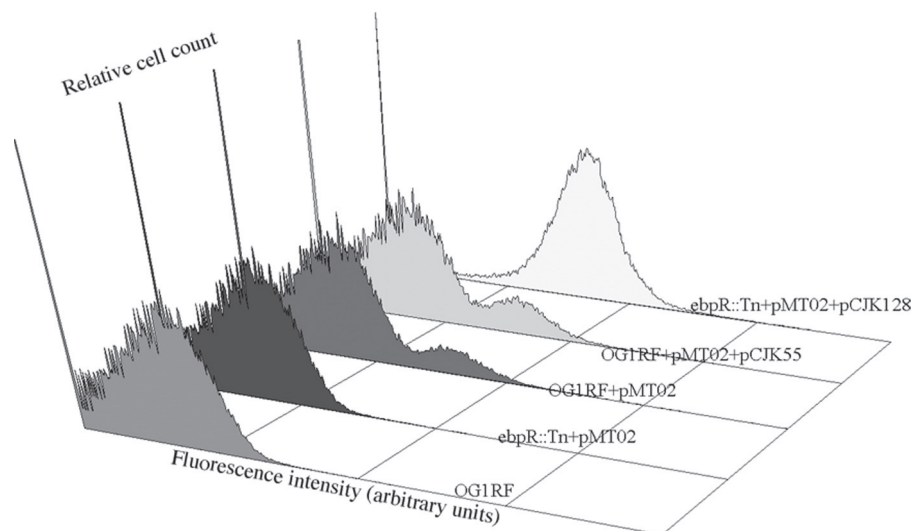


FIG 2 Activity of P_{ebp} -*gfp* in *E. faecalis*. Strains were grown in GM17 (in the presence of nisin in case the strain carries pCJK derivatives) for 24 h, and samples were analyzed by flow cytometry. For strain and plasmid descriptions, see Table 1.

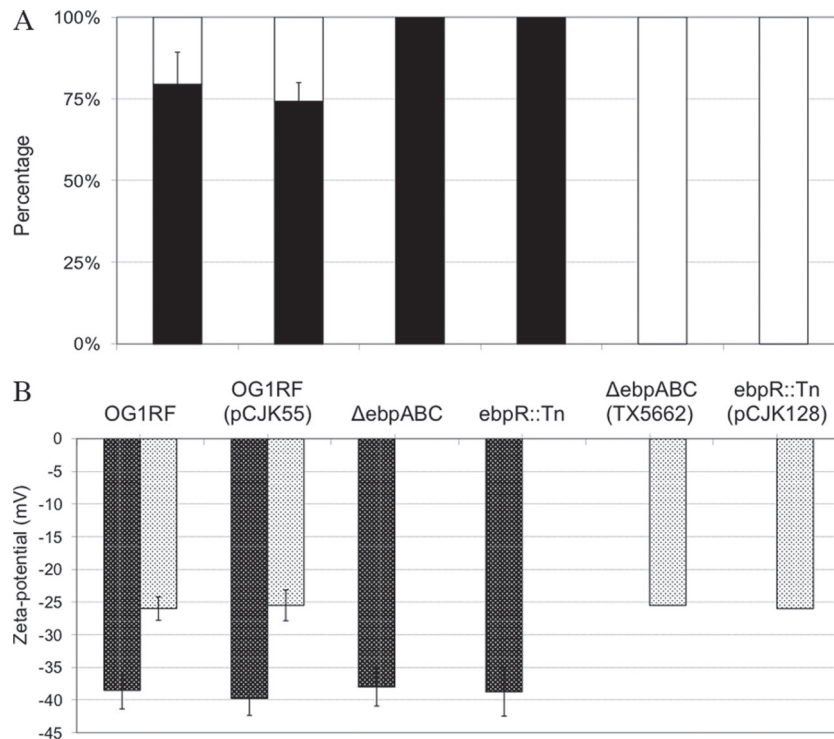


FIG 3 Percentage distributions (A) and magnitude of zeta potential (B) of subpopulations of *E. faecalis* strains. All strains except Δ ebpABC carried pMT02 and were grown in GM17 (with 25 ng/ml nisin in case the strain carries pCJK derivatives) for 24 h after which samples were analyzed. In both panels A and B, zeta potentials were determined in 10 mM potassium phosphate buffer (pH 7.0). Error bars are the standard deviations over three experiments in which at least 100 bacteria were examined in each experiment. The most negative population is indicated by a stippled black bar and the least negative population by a stippled white bar.

zeta potential (Fig. 3A). The most negative subpopulation, approximately 80% of the total, had an apparent zeta potential of around -38 mV, whereas the cells in the least negative subpopulation (20% of the total) have an apparent zeta potential of approximately -26 mV (Fig. 3B).

Deletion or overexpression of *ebpR* in *E. faecalis* results in homogeneous populations with respect to apparent zeta potential. Using the mariner transposon EfaMarTn on plasmid pCJK55, several mutations in the *ebp* locus were obtained in *E. faecalis* OG1RF, such as in 1D21 (the *ebpR*::Tn strain) as described previously (15). The EfaMarTn transposon contains a *gfp* gene, but it is not functional in most insertion mutants, including the strain carrying *ebpR*::Tn (15); thus, we could use pMT02 to examine the activity of P_{ebp} -*gfp* in this strain. Plasmid pCJK55 did not affect zeta potential heterogeneity, as OG1RF(pMT02; pCJK55) had a zeta potential distribution similar to that of OG1RF(pMT02) (Fig. 3A). The *E. faecalis* OG1RF *ebpR*::Tn mutant was shown to have a defect in biofilm formation (15). In addition, a clean *ebpR* deletion mutant, *E. faecalis* Δ ebpR, had lost its pilus-producing ability and primary adherence capability (3). No GFP signal was detected in cells of the strain carrying *ebpR*::Tn(pMT02), in agreement with the role of EbpR as a transcriptional activator of the *ebp* operon (see Fig. 2). In plasmid pCJK128, the *ebpR* gene is cloned under the control of the nisin-inducible promoter P_{nisA} (15). The *ebpR* overexpression strain OG1RF(pCJK128) and the complementation strain carrying *ebpR*::Tn(pCJK128) produce more pili and show enhanced biofilm formation relative to the wild type (3, 15). We introduced pMT02 in the EbpR complementation strain carrying

ebpR::Tn(pCJK128) to monitor P_{ebp} -driven GFP expression. Almost all cells of the *E. faecalis* strain carrying *ebpR*::Tn(pMT02; pCJK128) produced GFP (Fig. 2).

To examine the role of EbpR in the distribution of apparent zeta potential in *E. faecalis*, we measured this parameter in cultures of the *E. faecalis* strain carrying *ebpR*::Tn(pMT02) and the nisin-induced EbpR overexpression strain carrying *ebpR*::Tn(pMT02; pCJK128). Interestingly, both strains had lost the zeta potential heterogeneity seen in their isogenic parent OG1RF(pMT02) (Fig. 3B) and the control, the noninduced strain carrying *ebpR*::Tn(pMT02; pCJK128) (data not shown). The *ebpR*::Tn mutant had a zeta potential of -38 mV (Fig. 3B), which was similar to that of the most negative subpopulation of OG1RF. On the other hand, EbpR overexpression resulted in a population with a homogeneous apparent zeta potential of -26 mV, a value similar to that of the least negative subpopulation of OG1RF.

Deletion or overexpression of *ebpABC* results in homogeneous cultures with respect to apparent zeta potential. To examine the involvement of the EbpABC pilus of *E. faecalis* in apparent zeta potential, a clean knockout mutant lacking *ebpABC*, strain OG1RF Δ ebpABC (21), was used. Absence of EbpABC resulted in a homogeneous population of cells with an apparent zeta potential of -38 mV (Fig. 3), which was similar to that of the most negative subpopulation of its parent OG1RF. The complementation mutant OG1RF Δ ebpABC containing a plasmid in which *ebpABC* genes are cloned behind the constitutive P2 promoter of pAT392 (21), strain TX5662, formed a homogeneous population with an

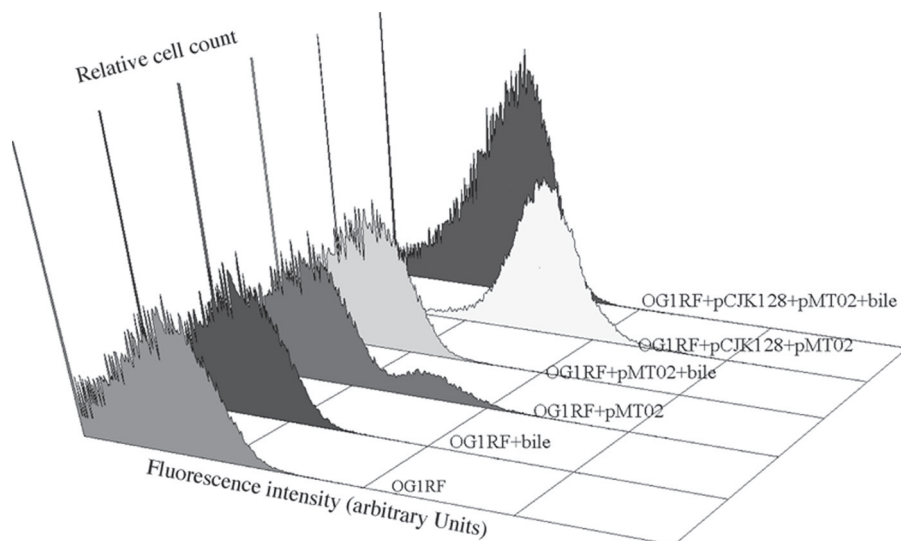


FIG 4 Effect of bile on P_{ebp} -gfp expression in *E. faecalis*. Strains were grown in GM17 at 37°C with and without bile for 24 h by shaking at 100 rpm, and samples were analyzed in a flow cytometer. For strain descriptions, see Table 1.

apparent zeta potential of -27 mV (Fig. 3), a value similar to that of the least negative subpopulation of OG1RF.

Expression driven by P_{ebp} is inhibited by ox bile. Ox bile in the growth medium influences the apparent zeta potential of certain *E. faecalis* strains, which ultimately affects biofilm formation (28). Thus, the effect of ox bile on the activity of the *ebp* promoter was examined by measuring GFP expression in strains carrying pMT02. The addition of ox bile (40 mg ml^{-1}) in these experiments had no effect on the pH of the medium or the growth rate of the strains (data not shown). While OG1RF(pMT02), a heterogeneous strain with respect to zeta potential, produces a large population of nonfluorescent cells and a smaller population of fluorescent cells, P_{ebp} -gfp activity was abolished when these cells were grown in the presence of ox bile (Fig. 4).

EbpR is the activator of P_{ebp} . The strain carrying *ebpR*::Tn(pMT02) does not produce a GFP signal (see above). Therefore, to examine whether EbpR plays a role in the effect of ox bile on P_{ebp} expression we used the EbpR overexpression strain OG1RF(pMT02; pCJK128). The strain was grown in the presence or absence of bile. Figure 4 shows that bile has a negative effect on P_{ebp} activity. Bile did not induce autofluorescence in these experiments (see controls OG1RF and OG1RF + bile in Fig. 4).

DISCUSSION

The increase in antibiotic resistance in bacteria has resulted in a search for alternative immunotherapeutic targets. Among such factors, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which are implicated in initiating infections such as endocarditis, have drawn considerable attention lately because they are commonly present in opportunistic pathogens (20). A recently recognized MSCRAMM that is important for biofilm formation in *E. faecalis* is the endocarditis and biofilm-associated pilus Ebp (20). The *ebp* operon is highly conserved in *E. faecalis* (19). Ebp pili mediate adherence to platelets, fibrinogen, and collagen, suggesting that they are involved in endocarditis, urinary tract colonization, or infections (19, 21).

Here, we report on the involvement of Ebp pili in the previously observed culture heterogeneity in zeta potential in axenic cultures of *E. faecalis*. We show that culture heterogeneity with respect to zeta potential, as described by van Merode et al. (28, 29), is correlated to heterogeneity in the presence of Ebp pili (20) on the surface of the bacterium. Deletion of the pilus structural genes *ebpAB* or the gene of the activator of the operon, *ebpR*, in a heterogeneous zeta potential *E. faecalis* strain results in cultures that are homogeneous with respect to apparent zeta potential. Homogeneous nonpilated cultures, like those from the OG1RF *ebpR*::Tn or $\Delta ebpABC$ mutant, have a zeta potential similar to that of the most negative subpopulation of OG1RF. In contrast, overexpression of *ebpR* or *ebpABC*, both of which are known to lead to an increase in pilus production in *E. faecalis* (3, 21), results in homogeneous cultures in which all cells have an apparent zeta potential similar to the least negative subpopulation of OG1RF.

We previously showed that heterogeneity in apparent zeta potential is a common feature of clinical *E. faecalis* isolates (29) and is correlated with the ability to form extensive biofilms (28). The *E. faecalis* mutants that overexpress *ebpABC* or *ebpR* all showed homogeneous populations with respect to zeta potential (this work) and have previously been shown to form extensive biofilms (3, 15). Therefore, it can be deduced that the presence of pili on the cell surface is more determinant for extensive biofilm formation than heterogeneity in apparent zeta potential.

The apparent zeta potential is derived from the electrophoretic mobility of individual bacteria. Zeta potentials are usually considered a measure for surface charge but can also be related to softness of particles. Softness of bacterial particles is dictated by surface structures such as pili that affect the width of the Stern layer. In a previous study, we showed that the heterogeneity in apparent zeta potential was pH dependent; at pH values lower than 5, the heterogeneity disappeared (29). If the apparent zeta potential is a measure of surface charge, it would be possible that aspartic acid or glutamic acid are ionized (neg-

TABLE 2 Net charges at different pH values predicted in the primary protein sequences of the structural proteins of the Ebp pilus^a

pH	Net charge		
	EbpA	EbpB	EbpC
4	82.7	45.4	47.5
5	9.3	16.8	4.5
7	-24.9	0.6	-17.3

^a The charges were calculated using Protein Calculator version 3.3 (<http://www.scripps.edu/~cdputnam/protcalc.html>) for each primary protein sequence retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/protein/>).

actively charged) at neutral pH while they are nonionized (neutral) at pH values below 5. Analysis of the pH dependency of the net charge of the primary protein sequences of the Ebp pilus (Table 2) revealed that EbpA displays significant negative charges at pH 7 (-24.9), while at pH 4, EbpA displays a significant positive charge (82.7). A similar but less significant charge behavior can be seen for EbpC while EbpB does not have a negative charge in the analyzed pH range. Alternatively, the absence of heterogeneity at low pH values could be caused by collapsing of pili onto the cell surface, which would effectively decrease the double layer and therefore the apparent zeta potential.

Previously, we have shown that heterogeneity in apparent zeta potential in *E. faecalis* is inhibited by bile. Specifically, the least negative population in a culture disappears upon exposure to bile. Expression of the *ebp* operon is similarly affected by bile as illustrated using the *P_{ebp}-gfp* reporter strains. Using these reporters, we show that the number of cells expressing *ebpABC* decreases upon exposure to bile and that this bile effect is communicated through the EbpR regulator. Another study showed that *ebp* locus expression was enhanced by the presence of bicarbonate with a consequential increase in the number of cells producing pili (4). Presently, it is unclear how bile affects the activity of EbpR on *ebp* expression.

The genetic basis for culture heterogeneity in *E. faecalis* remains uncertain. The *ebpRA* intergenic regions of several clinical isolates, both homogenous and heterogeneous with respect to apparent zeta potential (see Table 1), were sequenced, and the sequences were compared with those of the *ebpRA* regions of V583 and OG1RF. No differences in intergenic regions were observed (data not shown). It could be that there is some form of bistability in the expression of the *ebp* operon. In support of this notion, it is known that *ebpR* is autoactivated (3). A bistable regulation of Ebp pilus expression (and, thus, zeta potential heterogeneity) would mean that in most cells the levels of *ebpR* expression remain below a certain threshold level. However, if for some unknown reason the expression levels surpass this threshold, a positive feedback loop would amplify the signal, resulting in a strong increase of *ebpR* expression and, thus, pilus formation.

In conclusion, this study shows that heterogeneity in apparent zeta potential of *E. faecalis* is related to the presence or absence of Ebp pili. A better understanding of the regulation of *ebp* expression might allow for new approaches to thwart virulence and biofilm formation of *E. faecalis*. In this respect, the *P_{ebp}-gfp* fusion could be an important reporter to allow high-throughput screening of compound libraries to identify possible new antibiofilm agents, as illustrated in the present study using bile.

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